Regulation of tRNA methyltransferase activities by spermidine and putrescine

Inhibition of polyamine synthesis and tRNA methylation by α -methylornithine or 1,3-diaminopropan-2-ol in *Dictyostelium discoideum*

Michael MACH, Helga KERSTEN and Walter KERSTEN

Institute of Physiological Chemistry, University of Erlangen–Nürnberg, Fahrstrasse 17, 8520 Erlangen, Federal Republic of Germany

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Inhibitors of polyamine synthesis (α -methylornithine and 1,3-diaminopropan-2-ol) were used to study the relationship between polyamine synthesis and specific methylations of tRNA in *Dictyostelium discoideum* during vegetative growth. Polyamine concentrations were found to be 10 mM for putrescine, 1.6 mM for spermidine and 7 mM for 1,3-diaminopropane throughout the growth stage. On treatment of growing amoebae with α -methylornithine or with 1,3-diaminopropan-2-ol (each at 5 mM), the syntheses of putrescine, spermidine and 1,3-diaminopropane were arrested within 4 h. After polyamine synthesis had ceased, the incorporation of methyl groups into tRNA was considerably decreased under conditions that had no effect on the incorporation of uridine into tRNA, or on net syntheses of protein and of DNA. The following nucleosides in tRNA were concerned: 1-methyladenosine, 5-methylcytidine, 7-methylguanosine, 2-methylguanosine, N^2N^2 -dimethylguanosine and 5-methyluridine (ribosyl-thymine). The corresponding tRNA methyltransferases, determined in Mg^{2+} -free enzyme extracts, proved to be inactive unless polyamines were added. Putrescine and/or spermidine at concentrations of 10 mM or 1–2 mM respectively stimulate the transmethylation reaction *in vitro* to a maximal rate and to an optimal extent at exactly the same concentrations as found in vegetative cells. In contrast, 1,3-diaminopropane, which is formed from spermidine, does not affect the methylation of tRNA *in vitro* at physiological concentrations. Putrescine and/or spermidine stabilize the tRNA methyltransferases in crude extracts in the presence but not in the absence of the substrate tRNA. The results support the view that *S*-adenosylmethionine-dependent transmethylation reactions can be regulated by alterations of polyamine concentrations *in vivo*.

Polyamines are essential in pro- and eu-karyotes for optimal growth and development. The organic cations stabilize cells, membranes, DNA and ribosomes, they associate with nucleic acids and proteins and stimulate the syntheses of DNA and of RNA. In cell-free systems they can replace Mg^{2+} in the aminoacylation or methylation reaction of tRNA, they influence ribosome-dependent steps of protein synthesis and enhance the fidelity of translation. In addition, polyamines have been shown to modulate the activities of several enzymes not involved in interactions with nucleic acids (for references see Jänne *et al.*, 1978). The described functional effects of polyamines are probably caused by the interaction of a particular polyamine with macromol-

ecules at well-defined sites, e.g. two molecules of spermidine or spermine are tightly bound to tRNA (Sakai & Cohen, 1976). Spermidine was found in well-defined regions of the three-dimensional structure of tRNA^{Phe} from yeast (Quigley *et al.*, 1978). The association of polyamines with ribosomes also seems to be a highly specific process (Cohen, 1980). Interactions of polyamines with enzymes have still to be elucidated, but advances in this field have been described (Pegg, 1979; Sakai *et al.*, 1979).

During the synthesis of polyamines *S*-adenosylmethionine decarboxylase produces *S*-methyladenosylhomocysteamine, from which spermidine synthase (aminopropyltransferase, EC 2.5.1.16) transfers the propylamine residue to putrescine or

spermidine to form spermidine or spermine respectively (Pegg & Williams-Ashman, 1969). *S*-Adenosylmethionine plays another vital role: it serves as a donor of methyl groups in almost all transmethylation reactions of tRNA (Fleissner & Borek, 1962).

The transfer of methyl groups from *S*-adenosylmethionine to tRNA was found to be enhanced by putrescine or spermidine when cell-free extracts from rat liver were used as source of the methylating enzymes and heterologous major tRNA species from *Escherichia coli* as substrates (Leboy, 1970; Leboy & Glick, 1976; Pegg, 1971, 1973). *In vivo*, however, the methyltransferases incorporate the methyl groups into tRNA precursors in a well-defined sequence (Schäfer *et al.*, 1973; Nishikura & De Robertis, 1981). Therefore the question was raised as to whether the observed effects of polyamines on the methylation of a heterologous substrate tRNA *in vitro* might reflect a functional role of polyamines in tRNA methylation *in vivo*.

In order to solve these questions, we have used *Dictyostelium discoideum* as a model system. Vegetative amoebae of this organism contain relatively high amounts of three polyamines, namely putrescine, spermidine and the unusual 1,3-diaminopropan-2-ol. α -Methylornithine inhibits the ornithine decarboxylase competitively (Mamont *et al.*, 1976), whereas 1,3-diaminopropan-2-ol probably causes the formation of the antienzyme of ornithine decarboxylase (Alhonen-Hongisto *et al.*, 1979).

Here we show that the inhibition of polyamine synthesis in *D. discoideum* significantly decreases the intracellular methylation of particular nucleosides in tRNA at concentrations that had almost no effect on the incorporation of labelled precursors into DNA, protein and tRNA. The corresponding *S*-adenosylmethionine-dependent tRNA methyltransferases respond to physiological concentrations of putrescine and/or spermidine *in vitro*; they are stabilized by polyamines in the presence of tRNA.

Experimental

Chemicals

L-[methyl- ^3H]Methionine (sp. radioactivity 12 Ci/mmol), [^{14}C]uridine (58 Ci/mmol), [^3H]uridine (25 Ci/mmol), [^3H]thymidine (18.7 Ci/mmol), tritiated amino acid mixture (TRK. 440) and *S*-adenosyl-L-[methyl- ^{14}C]methionine (59 Ci/mmol) were purchased from The Radiochemical Centre Amersham, Braunschweig, Germany. [terminal-methylenes- ^3H]Spermidine trihydrochloride was

from NEN, Dreieich, Germany. Putrescine dihydrochloride, spermidine trihydrochloride and spermine tetrahydrochloride were obtained from Sigma, München, Germany. 1,3-Diaminopropane and 1,3-diaminopropan-2-ol were purchased from Fluka A.G., Buchs, Switzerland. DL- α -Methylornithine was generously given by the Centre de Recherche Merrell International (Strasbourg, France). Nuclease P_i was obtained from Sigma and alkaline phosphatase from Boehringer, Mannheim, Germany. All other chemicals were of analytical grade and were obtained from Merck, Darmstadt, Germany.

Organism and culture conditions

Dictyostelium discoideum, strain AX-2, kindly supplied by Dr. G. Gerisch (Max-Planck-Institut, Martinsried, Germany), grows in axenic medium (Watts & Ashworth, 1970) with a generation time of 8 h. Amoebae were usually grown at 22°C to a density of 2×10^6 – 4×10^6 cells/ml in Erlenmeyer flasks in a New Brunswick rotary shaker. The cells were harvested by centrifugation at 3000g for 3 min and washed twice with 'phosphate buffer' (2 mM- Na_2HPO_4 /14.7 mM- KH_2PO_4 , pH 6.7). For analyses of polyamines, RNA and protein, cells were harvested at 22°C, and for all other experiments at 4°C.

Preparation of polyamine- and tRNA-free extracts

Cells were harvested at 4°C and homogenized immediately for 5–10 min with 25 g of Alcoa A-305 (Serva) per 10 g wet wt. The homogenate was extracted with 15 ml of buffer I [10 mM-Tris/HCl (pH 7.5)/10 mM- MgCl_2 /0.1 mM- β -mercaptoethanol/10% (v/v) glycerol]. The extract was centrifuged for 10 min at 5000g and the supernatant for 45 min at 30000g. To the S-30 extract KCl was added to a final concentration of 0.2 M. Nucleic acids were separated from proteins by chromatography of the S-30 fraction on a DEAE-cellulose column (3 cm $\times$ 10 cm). Proteins were eluted with buffer II (buffer I + 0.2 M-KCl) and precipitated with $(\text{NH}_4)_2\text{SO}_4$ at 85% saturation. The precipitate was collected by centrifugation (30000g, 20 min), dissolved in 1 ml of buffer II, dialysed against 3×200 vol. of buffer III (buffer I without MgCl_2) and stored at -80°C . The protein content of the extracts determined by the micro-biuret method (Doetsch & Gadsden, 1973) was 10–15 mg/ml. An amount of extracts corresponding to 200 μg of protein does not contain polyamines in detectable amounts.

Analyses of polyamines, proteins and labelled RNA

Cells were harvested from 5–10 ml of culture at cell densities as indicated in the appropriate Table and Figure legends. The cells were extracted with 2×1 ml of 10% (w/v) trichloroacetic acid per 100 mg wet wt. by shaking at 4°C for 60 min. The

precipitate was removed by centrifugation (30000g for 5 min) and used for analyses of RNA and protein. From the combined supernatants 50–150 μ l were used for polyamine analysis, exactly as described previously (Mach *et al.*, 1981). Labelled RNA (labelling is described in the appropriate Table and Figure legends) was hydrolysed in the precipitate with 200 μ l of trichloroacetic acid at 90°C for 15 min, and was dissolved in 5 ml of toluene-based scintillation mixture (Zinser, Germany) and counted for radioactivity. The residual pellet was dissolved in 100 μ l of 1 M-NaOH at 60°C and protein was determined by the micro-biuret method.

DNA and protein syntheses

The net rates of DNA and protein syntheses were measured by adding 50 μ Ci of [3 H]thymidine or 50 μ Ci of 3 H-labelled amino acid mixture to 2 ml of cell suspension at cell densities of 2×10^6 /ml. After time intervals of 1, 3, 5 and 7 h 80 μ l samples were pipetted on to a Whatman 2MM filter (25 mm diameter) and precipitated for 1 h in 10% (w/v) trichloroacetic acid at 4°C. The filter discs were washed for 1 min each with 5% (w/v) trichloroacetic acid, ethanol and diethyl ether at 4°C. Radioactivity of the paper discs was analysed in toluene scintillation mixture.

Purification of tRNA by electrophoresis

Cells were grown and labelled with [3 H]uridine or L-[methyl- 3 H]methionine as described in the legend of Table 2. Cells from 5 ml of culture were suspended in 500 μ l of TMS buffer [10 mM-Tris/HCl (pH 7.5)/10 mM-MgCl₂/2 mM-Na₂S₂O₃]. Thereafter 500 μ l of TMS buffer, containing 1% (w/v) sodium dodecyl sulphate, was added, the suspension was stirred for 30 s and RNA extracted with phenol/TMS buffer (4:1, w/v) for 30 min at 4°C. The nucleic acids were precipitated from the aqueous phase with 2.5 vol. of ethanol, dissolved in electrophoresis buffer and applied to electrophoresis on a 10% polyacrylamide gel (Dingermann *et al.*, 1980).

Digestion of tRNA and analysis of methylated nucleosides

Electrophoretically purified tRNA (0.5 A_{260} unit) was dissolved in 20 μ l of 30 mM-ammonium acetate, pH 5.5, incubated for 2 min at 100°C and cooled in ice. Thereafter 2 μ l of 10 mM-ZnCl₂, 5 μ l of nuclease P₁ (1 mg/ml) and 10 μ l of alkaline phosphatase (6.8 mg/ml) were added. After incubation for 1 h at 37°C, 15 μ l of 0.5 M-Tris/HCl, pH 7.9, was added and the incubation continued for 1 h. The samples were used for high-performance-liquid-chromatography analysis in total.

High-performance-liquid chromatographic analysis of the methylated nucleosides was performed as described by Davis *et al.* (1979), with the

following modifications: buffer A, 0.1 M-NH₄H₂PO₄, pH 5.1; buffer B, 0.1 M-NH₄H₂PO₄, pH 5.1, + 40% (v/v) methanol. The two buffers were mixed as follows: 0–20 min, 100% A; 20–35 min, 90.4% A/9.6% B; 35–50 min, 81.2% A/18.8% B; 50–80 min, 18.8–100% B.

tRNA methyltransferase assay and analysis of the methylated products

The methylation assay (final volume 160 μ l) contained 3.3 mM-triethanolamine hydrochloride, pH 8.0, 0.55 mM- β -mercaptoethanol, 0.4 μ M-S-adenosyl-L-[methyl- 14 C]methionine (10.9 Ci/mol), 0.1 A_{260} unit of tRNA and enzyme extract corresponding to 200 μ g of protein. The mixture was incubated at 37°C or 25°C. At indicated times 50 μ l samples were pipetted on to a Whatman 2MM filter (25 mm diameter); the tRNA was precipitated and the discs were washed and counted for radioactivity as described above.

For analyses of the methylated nucleosides 0.2 A_{260} unit of tRNA was methylated with S-adenosyl-L-[methyl- 14 C]methionine (sp. radioactivity 29 Ci/mol). The reaction was stopped after 4 h by adding 160 μ l of phenol/chloroform (1:1, v/v). The tRNA was isolated as described by Dingermann *et al.* (1979). Analysis of nucleotides was performed as described by Silberklang *et al.* (1979). Bulk tRNA from *E. coli* IB-5 was used as a substrate and was prepared as described by Kersten *et al.* (1981).

Results

Polyamine concentrations and spermidine metabolism during vegetative growth

The myxamoebae of *Dictyostelium discoideum* strain AX-2 grow vegetatively in axenic medium in the presence of adequate food supply as independent cells with a generation time of 8 h. For analyses of polyamines and protein the amoebae were harvested at cell densities of 1.5×10^6 – 7.2×10^6 /ml. During exponential growth the amount of protein per cell remained constant. In each of eight different cell cultures 5.1 ± 0.71 (s.d.) mg of protein/ 10^8 cells was found. Three polyamines occur in vegetative amoebae, namely putrescine, spermidine and 1,3-diaminopropane (Table 1), putrescine in about 5-fold molar excess over spermidine and 1,3-diaminopropane at an unusually high concentration. The results agree well with those of North & Turner (1978), who analysed the polyamines of *Dictyostelium* by electrophoresis. Spermine, a polyamine of higher eukaryotes, was not detectable in our analysis and may occur in trace amounts.

In bacteria 1,3-diaminopropane can be derived from spermidine by cleavage by polyamine oxidase (Morgan, 1980). When radioactive spermidine, labelled at the terminal methylene groups, was added

Table 1. Polyamine concentrations in vegetative amoebae of *Dictyostelium discoideum*

Amoebae were grown exponentially in axenic medium to cell densities of 1.5×10^6 – 7.2×10^6 cells/ml. Cells from 5 ml or 10 ml of culture were harvested by centrifugation and washed twice with 5 ml of phosphate buffer (0.0167 M, pH 6.7). The polyamines were extracted with trichloroacetic acid. About 50–150 μ l of the combined extracts was applied to the columns and the polyamines were analysed as described by Mach *et al.* (1981). From the remaining precipitate nucleic acids were extracted by hydrolysis with trichloroacetic acid at 90°C. The residual protein was dissolved in NaOH at 60°C and determined by the micro-biuret method. The polyamine values are referred to 1 mg of protein. The results are means $\pm$ S.D. for eight determinations. For technical details see the Experimental section.

Polyamine	Content	
	(nmol/mg of protein)	(mM/cell)
Putrescine	112.3 $\pm$ 8.6	10.0
Spermidine	25.7 $\pm$ 2.5	1.6
1,3-Diaminopropane	64.0 $\pm$ 10.1	7.0

to growing amoebae and incubation continued for 4 h the radioactive label was found in 1,3-diaminopropane (Fig. 1). This result shows that *D. discoideum*, like bacteria, contains an enzyme that converts spermidine into 1,3-diaminopropane and 4-aminobutyraldehyde.

Inhibition of polyamine synthesis by α -methylornithine or 1,3-diaminopropan-2-ol

For each of the inhibitors the appropriate concentrations were evaluated that lead to a cessation of polyamine synthesis, but allow DNA and protein synthesis to proceed for at least one generation (8 h). At cell densities of about 2×10^6 /ml, the inhibitors were added. Concomitantly samples of the cell cultures were labelled with 3 H-labelled amino acids or with [3 H]thymidine. The incorporation of thymidine into DNA or of amino acids into proteins and the polyamine concentrations were determined during the following 8 h of incubation. The net syntheses of DNA and of protein remained almost unaffected for 8 h with both inhibitors at concentrations from 2 to 5 mM (results not shown).

On treatment of the amoebae with α -methylornithine the concentrations of 1,3-diaminopropane and of spermidine still increased during the first 4 h of treatment, that of spermidine even more than in the untreated control. This increase in spermidine was found in repeated experiments and can be explained by the inhibitory effect of α -methylornithine on the turnover of *S*-adenosylmethionine decarboxylase (McCann *et al.*, 1977; Mamont *et al.*,

Fig. 1. Formation of 1,3-diaminopropane from spermidine in vegetative amoebae

During vegetative growth, at a cell density of 10^6 cells/ml, 5 ml of culture was labelled with 10 μ Ci of [terminal-methylenes- 3 H]spermidine (43 Ci/mmol). After 4 h of incubation a 1 ml sample was removed: the cells were harvested by centrifugation and washed twice with 'phosphate buffer'. The polyamines were extracted as described in the legend of Table 1. Polyamines were analysed by ion-exchange chromatography and detected fluorimetrically by reaction with *o*-phthalaldehyde. For technical details see the Experimental section. —, Relative fluorescence; ----, radioactivity (c.p.m.). Abbreviations: DAP, 1,3-diaminopropane; Put, putrescine; Spd, spermidine.

1978). On further incubation the net syntheses of the polyamines were blocked and their concentrations remained constant or even decreased slightly (Fig. 2a). In the presence of 1,3-diaminopropan-2-ol (5 mM) the syntheses of all three polyamines ceased within 1 h (Fig. 2b).

Effects of α -methylornithine or 1,3-diaminopropan-2-ol on net synthesis and overall methylation of RNA

Either of the inhibitors was added to growing amoebae and the incubation continued until the synthesis of polyamines had ceased (4 h). At this time a cell culture was double-labelled with [14 C]-uridine and L-[methyl- 3 H]methionine. After pretreatment with α -methylornithine or with 1,3-diaminopropan-2-ol at the appropriate concentration, the incorporation of uridine into total RNA was inhibited by about 10% or 20% respectively. However, in cells pretreated with α -methylornithine the incorporation of radioactivity from methionine into total RNA was decreased by about 50%, and by about 70% in cells pretreated with 1,3-diaminopropan-2-ol (Fig. 3).

Fig. 2. Inhibition of polyamine synthesis by α -methylornithine or 1,3-diaminopropan-2-ol

During vegetative growth, at cell densities of 10^6 /ml, either of the inhibitors (a, α -methylornithine; b, 1,3-diaminopropan-2-ol) was added to a 100ml culture to a final concentration of 5mM. Samples (10ml) were removed after 2, 4, 6, 8 and 10h, the polyamines were extracted and analysed as described in the legend of Table 1. ●, ○, Spermidine; ▲, △, 1,3-diaminopropane; ■, □, putrescine. Open symbols represent untreated control; closed symbols represent treatment with either inhibitor.

Fig. 3. Influence of α -methylornithine or 1,3-diaminopropan-2-ol on net incorporation of $[^{14}\text{C}]$ uridine (a) or $[^3\text{H}]$ methyl groups (b) from methionine into total RNA

To 5ml of vegetatively growing amoebae (cell density 2×10^6 /ml), inhibitor was added to a final concentration of 5mM and the incubation continued further for 4h. Thereafter the cells were labelled for 4h with both $20\mu\text{Ci}$ of $[^{14}\text{C}]$ uridine (58Ci/mol) and $100\mu\text{Ci}$ of $\text{L-}[^3\text{H}]\text{methyl-methionine}$ (12Ci/mmol). Samples (1ml each) were removed at indicated times and the incorporation of radioactive precursors into RNA was determined. Polyamines were analysed concomitantly. For details see the Experimental section. ○, Control; □, culture with α -methylornithine; △, culture with 1,3-diaminopropan-2-ol.

In a second experiment of this series the specific radioactivities of ^{14}C and ^3H derived from $[^{14}\text{C}]$ uridine and from $[^3\text{H}]\text{methyl-methionine}$ respectively

were determined in tRNA from controls and from cultures that had been treated with 1,3-diaminopropan-2-ol. The incorporation of $[^{14}\text{C}]$ uridine into

tRNA was not affected by 1,3-diaminopropan-2-ol under conditions that decrease the incorporation of [^{14}C]uridine into total RNA by 10–20% (Table 2). However, the incorporation of ^3H from methionine was decreased. This result was obtained in two independent experiments.

Inhibition of the methylation of specific nucleosides in tRNA by 1,3-diaminopropan-2-ol

At 4 h after pretreatment of vegetative amoebae with 1,3-diaminopropan-2-ol (5 mM), treated cells and corresponding controls were labelled for 4 h with

Table 2. *Influence of 1,3-diaminopropan-2-ol (DAP-OH) on the synthesis and methylation of tRNA*

To 5 ml of vegetatively growing amoebae (cell density $2 \times 10^6/\text{ml}$) the inhibitor was added at a final concentration of 5 mM. The cells were incubated further for 4 h. Thereafter the cells were labelled with 1 mCi of L-[methyl- ^3H]-methionine (12 Ci/mmol) or 0.25 mCi of [^3H]uridine for 4 h. The cells were harvested and washed twice with an equal volume of phosphate buffer (0.0167 M, pH 6.7). Total RNA was extracted, the tRNA species were separated by electrophoresis, eluted, and the specific radioactivities of [^3H]uridine or [^3H]methyl groups determined (for technical details see the Experimental section).

Label	Culture conditions	Yield of tRNA (A_{260} unit)	Sp. radioactivity (c.p.m./ A_{260} unit)
^3H]Uridine	Control	0.24	1.2×10^5
	+DAP-OH	0.28	1.3×10^5
L-[methyl- ^3H]Methionine	Control	0.31	4.5×10^4
	+DAP-OH	0.25	2.7×10^4

Fig. 4. *Inhibition of the methylation of specific nucleosides in Dictyostelium tRNA by 1,3-diaminopropan-2-ol or α -methylornithine*

To 10 ml of vegetatively growing amoebae (cell density $2 \times 10^6/\text{ml}$) the inhibitor was added at a concentration of 5 mM and the incubation continued further for 4 h. Treated cells and a corresponding control were labelled with 1 mCi of L-[methyl- ^3H]methionine (12 Ci/mmol); 4 h later cells were harvested, tRNA was extracted, purified, digested to nucleosides and analysed by high-performance liquid chromatography by the method of Davis *et al.* (1979), with slight modifications as described in the Experimental section. ^3H -labelled nucleosides: —, control; ----, treated cells. For technical details see the Experimental section. Both inhibitors gave identical elution profiles; the experiment with 1,3-diaminopropan-2-ol is shown as representative. Abbreviations: m ^3C , 3-methylcytosine; m ^1A , 1-methyladenosine; m ^5C , 5-methylcytosine; m ^1G , 7-methylguanosine; T, ribosylthymine; G, guanosine; m ^1G , 1-methylguanosine; m ^2G , 2-methylguanosine; A, adenosine; m ^2G , N^2,N^2 -dimethylguanosine.

Fig. 5. Influence of spermidine and putrescine on overall tRNA methyltransferase activities and capacities in cell-free extracts from *Dictyostelium discoideum*

A portion (0.1 A_{260} unit) of tRNA from *E. coli* strain IB-5 (lacking ribosylthymine at position 54) was methylated with S-30 extracts corresponding to 200 μ g of protein and 20 nmol of *S*-adenosyl-L-[methyl- 3 H]methionine (sp. radioactivity 10.9 Ci/mol). The S-30 extract was freed from Mg^{2+} by

L-[methyl- 3 H]methionine. tRNA species were isolated and digested and the nucleosides analysed by high-performance liquid chromatography (Davis *et al.*, 1979). The incorporation of [3 H]methyl groups into almost all of the methylated nucleosides was considerably decreased in tRNA from cells treated with 1,3-diaminopropan-2-ol or α -methylornithine (Fig. 4). The effect of the inhibitors on the methylation of specific nucleosides in tRNA was most pronounced for ribosylthymine and N^2N^2 -dimethylguanosine (40% inhibition). The other methylated nucleosides contain about 30% less radioactivity than those obtained from the control tRNA. The methylation of guanosine to 1-methylguanosine was inhibited by only 15%.

Two radioactive labelled peaks were found in tRNA digests from untreated controls which were absent from tRNA digests from treated cells (Fig. 4). The retention time of these nucleosides corresponded to guanosine or adenosine. Also in thin-layer chromatograms by the method of Rogg *et al.* (1976) or Silberklang *et al.* (1979) radioactivity derived from methionine was found in guanosine or adenosine spots. This result suggested that the methyl group of methionine is somehow metabolized in *Dictyostelium* and incorporated into the purine ring system, a biochemical pathway which is totally blocked by α -methylornithine or 1,3-diaminopropan-2-ol.

Enhancement and inhibition of tRNA methyltransferase activities by polyamines *in vitro*

Supposing that the polyamines regulate the transfer of methyl groups to tRNA by an interaction with the methylating enzymes, it is expected that enzymes from different sources respond *in vitro* to polyamines according to their specific intracellular concentration and distribution. In order to elucidate this question, *E. coli* tRNA was used as a substrate in transmethylation reactions with methyltransferases from *D. discoideum* and with the tRNA (adenine-1)-methyltransferase from *Bacillus subtilis*, described by Raettig *et al.* (1977).

tRNA containing at position 54 uridine instead of ribosylthymine was obtained from the *E. coli* mutant IB-5 (Björk & Isakson, 1970) and proved to be a more efficient substrate for the tRNA methyltransferases from *D. discoideum* than was the methyl-deficient tRNA from *E. coli* Meth⁻ Rel⁻ 168/21 (Fleissner & Borek, 1962). Since Mg^{2+} can counteract the effect of polyamines, the enzyme extracts were dialysed against Mg^{2+} -free buffer.

dialysis. (a), (b), Kinetics; (c), overall capacity: ●, reaction time 120 min; ○, reaction time 240 min. Spermidine (a) or putrescine (b) was added at the concentrations indicated on the curves.

Table 3. *Stability of tRNA methyltransferases in the presence of substrate and polyamines*
 tRNA ($0.1 A_{260}$ unit) from *E. coli* strain IB-5 was methylated with the enzyme extract as described in the legend of Fig. 5. The enzyme extract was preincubated under the conditions, and the reaction started, as described in the Table. The concentration of putrescine was 10 mM and that of spermidine 1 mM. Incubation temperature was 25°C. Abbreviation: Ado-Met, S-adenosylmethionine.

Assay	Start	Methyl groups incorporated into $1 A_{260}$ unit of tRNA after preincubation (nmol)			
		Time ...	30 min	60 min	90 min
Enzyme extract + tRNA	Ado-Met		0.38	0.38	0.38
Enzyme extract + Ado-Met	tRNA		0.4	—	—
Enzyme extract + putrescine	Ado-Met + tRNA		0.4	—	—
Enzyme extract + spermidine	Ado-Met + tRNA		0.4	—	—
Enzyme extract + tRNA + putrescine	Ado-Met		1.5	1.4	0.80
Enzyme extract + tRNA + spermidine	Ado-Met		1.4	1.5	0.85

Without added polyamines the methyltransferase from *D. discoideum* was almost inactive. Optimal overall tRNA methyltransferase activities were observed in the presence of 1–2 mM-spermidine or 10 mM-putrescine (Fig. 5). These are exactly the intracellular concentrations of polyamines in growing amoebae (Table 1). A combination of both polyamines does not further increase the overall enzyme activities. 1,3-Diaminopropane does not activate the tRNA methyltransferases at physiological concentrations.

The main product, formed with *Dictyostelium* extracts and *E. coli* tRNA as a substrate, is N^2N^2 -dimethylguanosine at optimal concentrations of spermidine or putrescine. About 20% of the total methylated products comprise 1-methylguanosine + 2-methylguanosine, about 10% 5-methylcytosine + 7-methylguanosine and 10–13% 1-methyladenosine. When the *E. coli* tRNA was used as a substrate for the *B. subtilis* (adenine-1)-methyltransferase, optimal activity of the enzyme was obtained in the presence of spermidine only and at a final concentration of 0.1 mM. According to our analysis spermidine is the sole polyamine present in *B. subtilis*, at an intracellular concentration of about 0.4 mM (M. Mach, unpublished work). This result supports the view that the effect of the polyamines on the transmethylation reaction is caused predominantly by an interaction of polyamines with the methylating enzymes.

Polyamines might stabilize the methylating enzymes or the enzyme–tRNA complex. In order to test this hypothesis, crude enzyme extracts from *D. discoideum* were preincubated for 30 or 60 min (a) in the absence of tRNA and polyamines, (b) in the presence of tRNA, (c) in the presence of polyamines and (d) in the presence of tRNA and polyamines. The overall activity of tRNA methyltransferases decreased progressively during 60 min of prein-

cubation under all conditions except in the presence of polyamines and the substrate. The enzymes remained stable for at least 60 min when tRNA and polyamines were present (Table 3).

Discussion

Three polyamines occur in *Dictyostelium discoideum*, putrescine, spermidine and 1,3-diaminopropane. The last is present at unusually high concentration. Spermine occurs only in trace amounts, if at all. This organism was thought to be a useful model system in which to evaluate the specificity and physiological significance of the stimulatory effects of polyamines on tRNA methylation by eukaryotic enzymes *in vivo*.

As shown in the present work, 1,3-diaminopropane is a metabolite of spermidine, probably formed by a polyamine oxidase that cleaves spermidine into 1,3-diaminopropane and 4-aminobutyraldehyde (Mamont *et al.*, 1976). In contrast with putrescine and spermidine, 1,3-diaminopropane has no effect on the methylation of tRNA by the tRNA methyltransferases from *Dictyostelium in vitro*. The physiological function of 1,3-diaminopropane in *D. discoideum* is not yet known. It is possible that this spermidine metabolite when present at a critical concentration regulates ornithine decarboxylase by inducing the synthesis of an antienzyme, a mechanism observed in mammalian cells (Pösö & Jänne, 1976; Mamont *et al.*, 1978).

Inhibitors of polyamine synthesis have been applied in various eukaryotic cell systems to create a partial polyamine deficiency and to evaluate the relationship between polyamine concentrations and the syntheses of macromolecules. In *D. discoideum* the competitive inhibitor of ornithine decarboxylase, α -methylornithine, at a concentration of 5 mM has almost no effect on DNA, RNA and protein

synthesis over a period of one generation time (8h). Polyamine synthesis, however, was blocked within 4h. This result agrees well with the observations by Abdel-Monem *et al.* (1974, 1975), who studied the inhibitory effect of α -methylornithine on rat hepatoma cells. The incorporation of labelled methyl groups into overall tRNA ceases when the blockage of polyamine synthesis becomes pronounced. Since the incorporation of uridine into tRNA was not inhibited, the methylation of tRNA during processing and maturation is predominantly affected. The qualitative analysis shows that the methylation of almost all nucleosides decreases.

A possible relationship between spermidine and RNA synthesis was already discussed by Cohen & Lichtenstein (1960). Since then several workers have reported results on a strict correlation between the synthesis of RNA and ribosomes and the synthesis of polyamines (for references see Russell & Durie, 1978). It has also been discussed for several years that a correlation might exist between polyamine concentrations and tRNA methyltransferase activities in various tissues, underlying growth changes, differentiation or neoplastic transformation. However, a strict relationship between polyamine concentrations and the methylation of specific nucleosides in tRNA has not been demonstrated so far.

In the present study we show that spermidine or putrescine stimulates the overall tRNA methyltransferase activities in enzyme extracts of *D. discoideum* to an optimal rate and extent at the intracellular concentration. A decrease of polyamines within the cells to about 50% causes an incomplete methylation of tRNA. The polyamine concentrations decrease in *D. discoideum* during development (North & Turner, 1978). We have shown that the total amount of ribosylthymine and other modified nucleosides decreases in total tRNA during development of *Dictyostelium* (Dingermann *et al.*, 1980; F. Pistel, unpublished work). The results support the view that polyamines are involved in the regulation of tRNA methyltransferases.

Alterations of tRNA methyltransferase reactions by changes in the concentrations of putrescine and spermidine may be caused by an interaction of the polyamines with the substrate tRNA or with the methylating enzyme. Only spermine and spermidine will bind to tRNA, whereas the diamines putrescine and cadaverine seem to have little effect on tRNA structure, as shown by n.m.r. studies with tRNA^{Phe} (Bolton & Kearns, 1977). The observed stimulation of tRNA methylation by putrescine strongly suggests that putrescine interacts with the enzyme or with the enzyme-tRNA complex rather than with the tRNA. Another experiment shows that the concentration of spermidine necessary to stimulate a transmethylation reaction with the *E. coli* tRNA as

substrate depends on the source of the enzyme. The *B. subtilis* enzyme and the *Dictyostelium* enzyme are stimulated optimally by their specific polyamines at their intracellular concentrations. The results presented here further show that the stimulation of the tRNA methyltransferase reaction *in vitro* is caused by a stabilization of the enzyme in the presence of a substrate tRNA. Therefore polyamines may be involved in the regulation of tRNA methyltransferases as they are in the regulation of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase (Canellakis *et al.*, 1978; Mamont & Danzin, 1981). Since polyamine-synthetic pathways and nucleic acid methylation share the common co-substrate *S*-adenosylmethionine, it is likely that the two metabolic routes have common regulatory features.

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References

- Abdel-Monem, M., Newton, N. R. & Weeks, C. E. (1974) *J. Med. Chem.* **17**, 447–451
- Abdel-Monem, M., Newton, N. R. & Weeks, C. E. (1975) *J. Med. Chem.* **18**, 945–948
- Alhonen-Hongisto, L., Pösö, H. & Jänne, J. (1979) *Biochim. Biophys. Acta* **564**, 473–487
- Björk, G. & Isakson, L. (1970) *J. Mol. Biol.* **51**, 83–100
- Bolton, P. H. & Kearns, D. R. (1977) *Biochim. Biophys. Acta* **477**, 10–19
- Canellakis, E., Heller, J., Kyriakidis, D. & Chen, K. (1978) in *Advances in Polyamine Research*, vol. 1 (Campbell, R., ed.), pp. 17–31, Raven Press, New York
- Cohen, S. S. (1980) *Proc. Int. Symp. Polyamines in Normal and Neoplastic Cells*, in the press
- Cohen, S. S. & Lichtenstein, J. (1960) *J. Biol. Chem.* **235**, 2112–2116
- Davis, G., Gehrke, C. & Kuo, K. (1979) *J. Chromatogr.* **173**, 281–298
- Dingermann, Th., Mach, M. & Kersten, H. (1979) *J. Gen. Microbiol.* **115**, 223–232
- Dingermann, Th., Pistel, F. & Kersten, H. (1980) *Eur. J. Biochem.* **104**, 33–40
- Doetsch, K. & Gadsden, H. (1973) *Clin. Chem.* **19**, 1170–1178
- Fleissner, E. & Borek, E. (1962) *Proc. Natl. Acad. Sci. U.S.A.* **48**, 1199–1203
- Jänne, J., Pösö, H. & Raina, A. (1978) *Biochim. Biophys. Acta* **473**, 241–293
- Kersten, H., Albani, M., Männlein, E., Praisler, E., Wurmbach, P. & Nierhaus, K. (1981) *Eur. J. Biochem.* **114**, 451–456
- Leboy, Ph. (1970) *Biochemistry* **7**, 1577–1583
- Leboy, Ph. & Glick, J. (1976) *Biochim. Biophys. Acta* **435**, 30–38

- Mach, M., Kersten, H. & Kersten, W. (1981) *J. Chromatogr.* **223**, 51–57
- Mamont, P. & Danzin, Ch. (1981) *Adv. Polyamine Res.* **3**, 123–137
- Mamont, P., Böhlen, P., McCann, P., Bey, P., Schuber, F. & Tardiff, Ch. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1626–1630
- Mamont, P., Duchesne, M. C., Grove, J. & Tardiff, Ch. (1978) *Exp. Cell Res.* **115**, 387–393
- McCann, P., Tardiff, Ch., Duchesne, M. C. & Mamont, P. (1977) *Biochem. Biophys. Res. Commun.* **64**, 336–341
- Morgan, D. M. (1980) in *Polyamines in Biomedical Research* (Gaugas, J. M., ed.), pp. 295–303, John Wiley and Sons, Chichester and New York
- Nishikura, K. & De Robertis, E. M. (1981) *J. Mol. Biol.* **145**, 405–420
- North, M. & Turner, R. (1978) *Microbios Lett.* **4**, 221–228
- Pegg, A. E. (1971) *Biochim. Biophys. Acta* **232**, 630–642
- Pegg, A. E. (1973) *Biochim. Biophys. Acta* **319**, 354–363
- Pegg, A. E. (1979) *J. Biol. Chem.* **254**, 3249–3253
- Pegg, A. E. & Williams-Ashman, H. G. (1969) *J. Biol. Chem.* **244**, 682–693
- Pösö, H. & Jänne, J. (1976) *Biochem. J.* **158**, 485–488
- Quigley, G., Teeter, M. M. & Rich, A. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 64–68
- Raettig, R., Kersten, H., Weissenbach, J. & Dirheimer, G. (1977) *Nucleic Acids Res.* **4**, 1769–1782
- Rogg, H., Brambilla, R., Keith, G. & Staehelin, M. (1976) *Anal. Biochem.* **48**, 172–198
- Russell, D. H. & Durie, B. G. (eds.) (1978) *Prog. Cancer Res. Ther.* **8**, 1–173
- Sakai, T. T. & Cohen, S. S. (1976) *Proc. Nucleic Acid Res. Mol. Biol.* **17**, 15–139
- Sakai, T. T., Hovi, Ch., Kano, K. & Oka, T. (1979) *Biochemistry* **18**, 5541–5548
- Schäfer, K., Altmann, S. & Söll, D. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3626–3630
- Silberklang, M., Gillum, A. M. & RajBhandary, U. L. (1979) *Methods Enzymol.* **59**, 58–109
- Watts, D. J. & Ashworth, J. M. (1970) *Biochem. J.* **119**, 171–174